Neutrophil Interaction with Emerging Oral Pathogens: A Novel View of the Disease Paradigm

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Introduction

Periodontitis is a multifactorial chronic inflammatory disease that affects the integrity of the periodontium (composed of the gingiva, periodontal ligament, cementum, and alveolar bone). The prevalence of the disease is high, affecting 42% of adults 30 years or older in the USA alone (Eke et al. 2018). Treatment includes deep cleaning, antibiotics, and in severe cases surgery (Douglass 2006). Unfortunately, these treatments are only efficacious in the short term because the infection almost always returns. This disease has also been associated with several comorbidities including rheumatoid arthritis and cardiovascular disease among other inflammatory conditions (Kebschull et al. 2010; Bingham and Moni 2013; Hajishengallis 2015). Novel culture-independent techniques have facilitated the identification of new bacterial species at periodontal lesions and induced a reappraisal of the microbial etiology of periodontitis (Costalonga and Herzberg 2014). The current model describing the etiology and pathogenesis for periodontitis is the polymicrobial synergy and dysbiosis (PSD) model (Hajishengallis 2013; Hajishengallis and Lamont 2012). The PSD model indicates that a perturbation of the symbiotic microbial community, associated with periodontal health, results in an increase in the diversity and microbial burden. This results in a dysbiotic microbial community that can adapt to and take advantage of the inflammatory environment to enhance bacterial fitness (Hajishengallis and Lamont 2012).

The host inflammatory response against this dysbiotic microbial community causes the destruction of the periodontium, and human neutrophils are the main phagocytic cell recruited to the periodontal pocket (Scott and Krauss 2012; Uriarte et al. 2016). Based on the published evidence, neutrophils control the indigenous microbial community by mounting an acute inflammation to preserve a healthy gingival tissue. However, neutrophil response against the dysbiotic microbial community results in a dysregulated inflammatory, response causing disease progression (Darveau 2009, 2010). Therefore, to maintain periodontal health, a fragile equilibrium between neutrophil recruitment and activation at the gingiva is essential.

The advance of new high-throughput technology has revealed that the etiology of periodontitis is more complex than the initial paradigm. High numbers of fastidious and "yet-to-be cultivated"

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G. N. Belibasakis et al. (eds.), *Oral Mucosal Immunity and Microbiome*, Advances in Experimental Medicine and Biology 1197, https://doi.org/10.1007/978-3-030-28524-1_12

taxons with strong correlation with disease progression have been identified by human oral microbiome studies (Kumar et al. 2003, 2005). Characterization of the pathogenic potential of these newly appreciated oral pathogens is just beginning to emerge (Hajishengallis and Lamont 2016). The ability of these emerging oral pathogens—as members of the dysbiotic community—to flourish at the gingiva suggests that the microorganisms developed mechanisms to evade or disable the innate immune system. In this chapter, we highlight the clinical studies that opened the Pandora box of the oral mucosa and revealed the presence of newly appreciated periodontitis-associated bacteria, basic description of neutrophil effector functions, and discuss the studies that describe the interactions between neutrophils and the newly appreciated oral pathogens.

Dysbiosis in Periodontitis: Revealing the Presence of Underappreciated Bacteria

The microbial shift observed in the gingival crevice from a symbiotic oral microbiota to a dysbiotic polymicrobial community is currently the most accepted paradigm to describe the onset and progression of periodontitis (Hajishengallis 2013; Hajishengallis and Lamont 2016; Lamont and Hajishengallis 2015). Initial studies at the turn of the twentieth century used the microbiological techniques available at that time to begin characterizing the etiological agents responsible for periodontitis (Socransky and Haffajee 1994). Those initial studies discovered *spirochetes*, *fusiforms,* and *streptococci* (Socransky and Haffajee 1994; Feres et al. 2004) present in periodontal pockets. Later, using microscopic techniques, Listgarten et al. (Listgarten 1976; Listgarten and Hellden 1978) showed presence of morphological differences in the composition of subgingival microbiota in subjects with different stages of oral diseases. The development of immunological techniques and DNA probes helped to identify the microbial species present in the subgingival pockets (Tsai et al. 2003). However, high-throughput analysis of microbial communities present in subgingival pockets became possible with molecular DNA-based technologies. Using the community fingerprinting techniques, like restriction fragment length polymorphism (Deng et al. 2008), or denaturing gradient gel electrophoresis (Anderson and Cairney 2004), variation and shift in the composition of the microbial community in periodontitis could be identified. The development of DNA–DNA checkerboard hybridization helped elucidated the specific association of oral bacteria with health and disease (Dahlen and Leonhardt 2006). Socransky et al. (1998) described the presence of five microbial communities in subgingival biofilms associated with health and different stages of periodontitis. The 16S rRNA approach has revolutionized the identification of bacterial taxa by classifying whether they are cultivable or "yet-to-be-cultivated," in a mixed population and showed diversity of the oral microbiota (Paster et al. 2001, 2006). Next-generation sequencing technologies further reformed the study of oral microbial diversity by providing the existence of underappreciated periodontal pathogens (Kumar et al. 2006; Dewhirst et al. 2010; Abusleme et al. 2013; Aas et al. 2005; Griffen et al. 2012). The pioneer traditional studies together with the more advanced 16S rRNA gene comparative analysis identified presence of approximately 700 predominant taxa in oral microbiome of which approximately 1/3 is "yet-to-be-cultivated" (Krishnan et al. 2017). About 400–500 taxa were reported in the subgingival crevice alone (Paster et al. 2001; Aas et al. 2005).

The initial dogma in periodontitis was that the microbial community shifted from gram-positive aerobic to gram-negative anaerobic phenotype as disease progressed (Berezow and Darveau 2011; Marsh 1994). The plaque analysis from periodontitis-diseased sites revealed the presence of *Porphyromonas gingivalis*, *Tannerella forsythia,* and *Treponema denticola* related with disease severity (Socransky et al. 1998; Socransky and Haffajee 2005) and the presence of *Aggregatibacter actinomycetemcomitans* associated with aggressive periodontitis (Feres et al. 2004). However, examination of

the microbial composition present in the gingival crevice by the use of more advance techniques, revealed the complexity of the microbial communities and challenged the dogma of disease progression associated with a shift from gram-positive to gram-negative. For example, high numbers of gram-positive bacterium, *Filifactor alocis,* are present in disease periodontal pockets while increased number of gram-negative uncultivated *Veillonella sp* oral clone X042 are found in healthy periodontal pockets have been reported (Kumar et al. 2006). Furthermore, a recent review compared 41 published studies, from 1999 till 2013, that showed an association between emerging periodontal organisms and periodontitis and concluded that 17 newly identified species that includes five gram-positive (*Eubacterium saphenum*, *Mogibacterium timidum*, *Peptostreptococcus stomatis*, *F. alocis* and *Enterococcus faecalis*), eight gram-negative, and four not-yet-cultivable group have a moderate association with the etiology of periodontists (Perez-Chaparro et al. 2014).

The biofilms associated with different stages of periodontitis have substantial overlap in the microbial composition, and the phylogenetic profile of active and non-progressing lesions have similar microbial communities, raising the question whether just composition of microbes or the functional activity of microbial community leads to dysbiosis (Yost et al. 2015; Solbiati and Frias-Lopez 2018). Recent metatranscriptome studies of the subgingival plaque were able to identify changes in functional activities linked to progression of periodontitis (Yost et al. 2015; Jorth et al. 2014; Duran-Pinedo et al. 2015). For example, alteration in potassium ion transport increased the virulence of the oral community and altered the immune response of gingival epithelium (Yost et al. 2015; Duran-Pinedo et al. 2015). The metagenome and metatranscriptome analysis of subgingival plaques revealed the presence of underappreciated periodontal pathogens (Solbiati and Frias-Lopez 2018; Dabdoub et al. 2016). In order to better comprehend the role of these emerging organisms in the onset and progression of the disease, it is essential to study their interaction with other microbes in the community as well as with host cells. In this book chapter, we will focus on the published studies of two organisms classified as putative periodontal pathogens, *F. alocis* and *Peptoanaerobacter stomatis*. We will briefly introduce the current microbiological knowledge about *F. alocis* and *P. stomatis*, followed by what we know about their interaction with neutrophils.

Filifactor alocis

F. alocis was first identified in 1985 from the gingival sulcus in gingivitis and periodontitis patients and named as *Fusobacterium alocis* (Cato et al. 1985). In 1999, this bacterium was reclassified in genus *Filifactor* (Jalava and Eerola 1999). *F. alocis* is a gram-positive obligate anaerobic rod, non-spore forming, asaccharolytic, utilizing specific amino acids including arginine, and a slow growing bacteria (Jalava and Eerola 1999; Aruni et al. 2015). Multiple studies have shown the high prevalence of *F. alocis* in subgingival plaques of periodontitis patients compared to absence or low number detected at healthy sites (Kumar et al. 2003, 2005, 2006; Perez-Chaparro et al. 2014). The incidence of *F. alocis* has been reported also in endodontic infections (Zhang et al. 2012; Siqueira and Rocas 2003; Siqueira et al. 2009) and peri-implantitis (da Silva et al. 2014). A study focusing on co-occurrence of oral pathogens in disease sites showed a positive correlation between *F. alocis* and eight oral pathogens including *P. gingivalis* and *T. forsythia,* suggesting possible synergistic interactions among them (Chen et al. 2015). It has been established that *F. alocis* can form biofilm close to apical and middle thirds of the gingival pockets in close proximity to soft tissue (Schlafer et al. 2010). In vitro studies have shown that *F. alocis* could interact with numerous oral bacteria and play significant role in community formation (Aruni et al. 2011; Wang et al. 2013). *F. alocis* co-cultured with *P. gingivalis* enhanced the biofilm formation (Aruni et al. 2011) forming heterotypic biofilm with enhanced *P. gingivalis* growth (Wang et al. 2013) and it also form biofilm with *Fusobacterium nucleatum* and *A. actinomycetemcomitans* (Wang et al. 2013).

F. alocis has an exceptional property of resistance to oxidative stress, with its growth stimulated under this condition (Aruni et al. 2011) providing an advantageous attribute to survive in the inflammatory environment that is a hallmark of periodontitis. Moreover, as an asaccharolytic bacterium, *F. alocis* amino acid requirement for its growth is likely fulfilled by the byproducts of the disrupted host tissue produced as a consequence of the chronic periodontal inflammation (Hajishengallis 2013; Aruni et al. 2011, 2015). Another important virulence property of *F. alocis* is the presence of different proteases, most of these proteases are membrane bound but an important collagen peptidase was identified in extracellular fraction that could play critical role in tissue destruction in periodontitis (Aruni et al. 2012). In vitro studies with epithelial cells show that *F. alocis* can adhere and invade in monoculture and this attribute is enhanced in presence of *P. gingivalis* (Aruni et al. 2011; Moffatt et al. 2011). *F. alocis* induces secretion of proinflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α from epithelial cells and promotes apoptosis by suppression of MEK1/2 and activation of caspase 3 (Moffatt et al. 2011). Thus, *F. alocis* has a wide array of potential virulence factors to survive in the hostile periodontitis environment.

Peptoanaerobacter stomatis

P. stomatis is a newly recognized perio-pathogen cultured from gingival plaques and is the first cultivable member of human oral taxon 081 (Sizova et al. 2012). It is classified as a novel genus and species within the family of *Peptostreptococcaceae* (Sizova et al. 2015). *P. stomatis* is a gram-positive, motile peritrichous rod often occurring in chains (Sizova et al. 2015). This organism is a strict anaerobe, non-spore forming, with a diameter of 0.5 to 0.7 μm and a length of 1.0 to 2.3 μm (Sizova et al. 2015). *P. stomatis* is present in high numbers in periodontal patients' oral biofilms (Kumar et al. 2005; Murphy and Frick 2013) and also reported in dentoalveolar abscesses and endodontic infections (Downes and Wade 2006). Characterization of the pathogenic potential of *P. stomatis* is in its infancy; future in vitro and in vivo studies will determine what role this organism plays in the dysbiotic microbial community present in periodontitis.

The Veil Disappeared and Revealed a Different Battlefield: Neutrophils coping with Emerging Oral Pathogens

The Neutrophil

If the integrity of the host is compromised, the first immune cell to respond and be recruited in vast numbers to the site of infection is the neutrophil (Ryder 2010). As primary effector cells of the innate immune system, neutrophils possess numerous strategies to locate, detain and kill microbes (Amulic et al. 2012; Kolaczkowska and Kubes 2013). Neutrophils have multiple killing mechanisms to eliminate both intracellular as well as extracellular microorganisms (Kolaczkowska and Kubes 2013; Nauseef 2007). In the context of oral mucosal immunity, neutrophils are found in large numbers in the gingival crevice and epithelium, entitling them as the major effector cell of the periodontium. The acute and regulated innate immune response, driven by neutrophils, is of critical importance to the maintenance of periodontal health in the host. Dysregulated recruitment of neutrophils to the gingiva, on the other hand, is associated with disease progression (Hajishengallis et al. 2016). In the inflamed periodontal tissue, chemotactic factors such as IL-8, as well as formylated bacterial-derived peptides such as fMLF, will be abundant and guide neutrophils from the blood vessels through the gingival tissue toward the periodontal pocket (Uriarte et al. 2016). Upon encounter with the offending microbe, the phagocytic process is initiated followed by high oxygen consumption through a process known as the respiratory burst with the generation of reactive oxygen species (ROS) within the bacteria-containing phagosome (Nauseef 2007). Activation of the NADPH

oxidase, a multicomponent enzyme, is responsible for ROS production (Babior et al. 2002; Belambri et al. 2018). Neutrophils have the capacity to tailor their response depending on the type of stimuli they encounter. Stimulation of neutrophils by a soluble stimuli, such as fMLF, triggers assembly and activation of the NADPH oxidase at the plasma membrane and release of superoxide anions towards the extracellular space. In contrast, if neutrophils encounter a particulate stimuli, for example a bacterium, assembly and activation of the NADPH oxidase will take place at the membrane of the bacteria-containing phagosome with release of superoxide anions inside the phagosome (Nauseef 2007, 2014; Babior et al. 2002).

As a member of the granulocyte family of leukocytes, neutrophils contain four different types of granules, which are differentiated based on their density, protein content, and on their functional response (Borregaard and Cowland 1997; Borregaard 2010). The different neutrophil granule subtypes can either be recruited to the bacteria-containing phagosome or stimulated to undergo exocytosis and release their matrix content to the extracellular environment (Borregaard et al. 2007). The hierarchical mobilization of neutrophil granules is driven by the strength of the stimuli required for its exocytosis. The weak stimuli induces mobilization of secretory vesicles, and increasing stronger stimulation is required to mobilize gelatinase, specific and azurophil granules, respectively (Nauseef and Borregaard 2014). The diverse repertoire of proteins and receptors present at the membrane of each granule subtype as well as within the granule lumen highlights the important role each granule plays in the different neutrophil responses during inflammation (Uriarte et al. 2008; Lominadze et al. 2005; Rørvig et al. 2013). The antimicrobial efficacy of neutrophils on intracellular as well as extracellular microorganisms is enhanced by the ability to combine both oxygen-dependent and independent mechanisms.

In 2004, Brinkmann et al. described a novel mechanism deployed by neutrophils to trap and kill microbes—neutrophil extracellular traps (NETs)—in which neutrophils extrude decondensed chromatin decorated with neutrophil granule proteins (Brinkmann et al. 2004). NETs can trap bacteria, due to electrostatic charge interactions, and have microbicidal effects due to the high concentration of localized antimicrobial peptides (Parker et al. 2012; White et al. 2016; Yipp et al. 2012; Fuchs et al. 2007; Brinkmann and Zychlinsky 2012). Depending on the stimuli that neutrophils encounter, production of intracellular ROS may or may not be required for NET formation (Parker et al. 2012; Fuchs et al. 2007; Vitkov et al. 2009; Remijsen et al. 2011; Pilsczek et al. 2010). Whether NET formation has beneficial or detrimental effects to the host in the presence of infection remains controversial (Brinkmann and Zychlinsky 2012; Barrientos et al. 2013; Cheng and Palaniyar 2013; Simon et al. 2013). In the context of periodontitis, both excessive and ineffective NET formation has been associated with disease progression (Cooper et al. 2013). Degradation of DNA, by the production of DNases, is a successful strategy utilized by microbial pathogens to escape trapping and killing by NETs (Vitkov et al. 2009; Buchanan et al. 2006; Beiter et al. 2006; Sumby et al. 2005; Porschen and Sonntag 1974; Rudek and Haque 1976; Dahlen et al. 1983). However, crevicular exudate outflow may inhibit optimal functioning of the bacterial DNases, and work in concert with NETs to clear pathogens from the oral cavity and prevent development of periodontitis (Vitkov et al. 2009). For a deeper understanding of neutrophil antimicrobial functions, the reader is referred to these review articles (Amulic et al. 2012; Nauseef 2007; Belambri et al. 2018; Borregaard 2010; Ley et al. 2018).

The traditional view of neutrophils as "pathogen busters" of innate immunity has changed over the past 20 years. Neutrophils role as regulators of the inflammatory and immune responses is well established by their capacity to transcribe, perform de novo synthesis, and release different cytokines and chemokines (Cassatella 1999; Tamassia et al. 2018; Tecchio and Cassatella 2016), as well as by exocytosis of granule proteins, primarily stored in azurophilic granules, with chemotactic activity toward neutrophils as well as other leuko-

cytes (Chertov et al. 2000). Neutrophils will produce and release an array of different cytokines and chemokines depending on the type of stimulation they encounter. The contribution of neutrophil-derived cytokines and chemokines, which are released at the local inflamed site, is significant in the amplification loop of the local immune response. For example, the release of CCL2 and CCL20 from neutrophils stimulated with IFN-γ and LPS promotes chemotaxis of Th17 cells in vitro (Pelletier et al. 2010). We can extrapolate those in vitro findings and speculate that infiltration of Th17 cells into the gingival tissue could be mediated, in part, by the chemokines locally produced by neutrophils exposed to the dysbiotic microbial environment. The ability of neutrophils to engage into cross talk relationships with other leukocytes highlights the primordial role that this professional phagocyte plays in modulation of the immune response.

Another example of pathogen manipulation of neutrophil effector functions during the inflammatory response is the activation of the triggering receptor expressed on myeloid cells 1 (TREM-1). Activation of TREM-1 by periodontal pathogens with concomitant increase levels of the soluble form sTREM-1 has been associated with enhanced cytokine production and periodontitis severity (Bostanci et al. 2013a). *P. gingivalis*, through its Lys and Arg-gingipains, induces activation of TREM-1 in human neutrophils and regulates both the release of sTREM-1 as well as its degradation according to the bacterium needs during the different stages of periodontitis progression (Bostanci et al. 2013b). More, in vivo studies are needed to gain better insights into the neutrophil-centered regulatory role during chronic inflammation to develop more efficacious therapies to combat chronic inflammatory diseases such as periodontitis. To deepen the knowledge of neutrophils as effector cells of the immune response, reading the following articles by Cassatella's group is recommended (Cassatella 1999; Tecchio and Cassatella 2016; Scapini and Cassatella 2014).

Neutrophils Coping with Two Emerging Gram-Positive Oral Pathogens

In chronic inflammatory infectious diseases, such as periodontitis, pathogenic microorganisms must subvert the immune response to survive in the human host. This outcome leads to disease due to the ability of the pathogenic bacteria to alter the normal neutrophil turnover by triggering neutrophil lysis, NETosis, or delaying neutrophil apoptosis after phagocytosis. As a result of this host–pathogen interaction, the tissue-damaging molecules released by neutrophils contribute to the generation of tissue breakdown products, which supports the nutritional needs of the asaccharolytic oral pathogens. The high number of *F. alocis* and *P. stomatis* found in subgingival pockets of periodontitis disease patients will suggest that these two organisms can subvert neutrophil antimicrobial mechanisms and successfully grow under inflammatory conditions.

Using the subcutaneous chamber model, *F. alocis* infection triggered a quick recruitment of neutrophils to the site of infection, the production of proinflammatory cytokines, and infected distal sites causing acute kidney damage primarily to the tubular epithelial cells (Wang et al. 2014). This in vivo study begins to point out the pathogenic profile of *F. alocis* and the ability of this organism to colonize other organs. In support of our initial observations, a recent study identified high content of *F. alocis* on the pleural fluid of a 65-year-old male patient hospitalized for thoracic empyema (Gray and Vidwans 2019). This case report study is the first one to show *F. alocis* presence outside the oral cavity and its association to another disease other than periodontitis. Our earlier report in mice and this recent case report in humans reveal that *F. alocis* can colonize distal organs outside the oral cavity.

In 2012, the pioneer study of Aruni et al. (Aruni et al. 2012) characterize the proteome map of *F. alocis* and described the presence of several putative virulence factors such as proteases, neutrophil-activating factor, and collagenases. In the diseased periodontal pocket, high

concentrations of complement proteins and its derivative peptides are found (Hajishengallis et al. 2019). Established periodontal pathogens evolved different complement evasion strategies to survive in the periodontal pocket (reviewed in (Hajishengallis and Lambris 2012, 2016). It has been described that *F. alocis* activates all the complement pathways and that C3b deposition is induced primarily by the alternative pathway and enhanced when the bacterium is grown in the planktonic form compared to biofilm growth (Jusko et al. 2016). Interestingly, *F. alocis* has a cytoplasmic enzyme, FACIN, that once present on the bacterial surface can inhibit activation of complement component 3 (C3) convertases (Jusko et al. 2016). FACIN also functions as an important enzyme involved in key metabolic pathways for bacterial growth, which will limit the possibility to generate an isogenic mutant. However, there is a need to perform genetic manipulation of *F. alocis* to generate isogenic mutants, since it will enable to gather better insight of the role that different putative virulence factors might play in microbial interaction and host response.

Manipulation of the host innate immune response allows periodontal pathogens, such as *P. gingivalis*, to drive in the periodontal pocket. *P. gingivalis* evades neutrophil antimicrobial killing by promoting a cross talk between TLR2/1 and C5a receptor that dismantles the antimicrobial signaling pathway while promotes the production of inflammatory mediators (Maekawa et al. 2014). The detection of high numbers of *F. alocis* in disease sites is an indication that this organism is able to subvert innate immunity. The initial characterization, from our laboratory, about how *F. alocis* interacts with neutrophils reveals that this putative oral pathogen can modulate neutrophil effector functions. *F. alocis* is recognized by TLR2 and triggers exocytosis of three of the four neutrophil granule subtypes through activation of both p38 MAPK and ERK1/2 (Armstrong et al. 2016). In addition, neutrophils that were challenged with either live or heat-killed *F. alocis* showed enhanced random and directed migration toward IL-8, an effect that was dependent on the bacteria-induced granule exocytosis (Armstrong et al. 2016). Human neutrophils efficiently internalized *F. alocis,* but the organism manages to subvert the antimicrobial response by inducing a minimal respiratory burst response and preventing phagosome maturation (Edmisson et al. 2018). In co-infection studies with either *Staphylococcus aureus* or *P. stomatis*, only the minimal ROS production will be localized in the *F. alocis*-containing phagosomes (Edmisson et al. 2018). Furthermore, only live *F. alocis* is able to inhibit granule trafficking to the phagosome to prevent phagosome maturation, which allows *F. alocis* to survive in neutrophils. *F. alocis,* independent of bacterial dose or time, does not induce NET formation, but pre-treatment of neutrophils with *F. alocis* significantly inhibits PMA-induced NETs (Armstrong et al. 2018). We recently reported that *F. alocis* signals primarily by TLR2/6 heterodimers and promotes release of neutrophil-derived cytokines and chemokines but to a lesser extend compared to *P. stomatis* (Vashishta et al. 2019). Figure 1a shows *F. alocis* ability to modulate neutrophil effector functions to promote bacterial survival.

In stark contrast, *P. stomatis* interaction with neutrophils results in hyperactivation of the professional phagocyte (Fig. 1b). Contrary to the response seen with *F. alocis,* when suspension neutrophils were exposed to *P. stomatis,* they display low phagocytic capacity toward this emerging oral pathogen. However, the low percent of *P. stomatis* that was engulfed by neutrophils was killed primarily by oxygen-independent mechanisms (Jimenez Flores et al. 2017). *P. stomatis* interaction with neutrophils results is both a strong respiratory burst response and significant exocytosis of the four neutrophil granule subtypes. In addition, *P. stomatis* is a strong priming agent of neutrophil respiratory burst response. Pre-treatment of neutrophils with *P. stomatis* significantly enhances fMLF-induced respiratory burst response similar to levels achieved by TNFα—a well-established neutrophil priming agent (Jimenez Flores et al. 2017). The ability of *P. stomatis* to prime neutrophils and to promote release of granule content will contribute to tissue breakdown and chronic inflammation, both features associated with periodontitis. Furthermore,

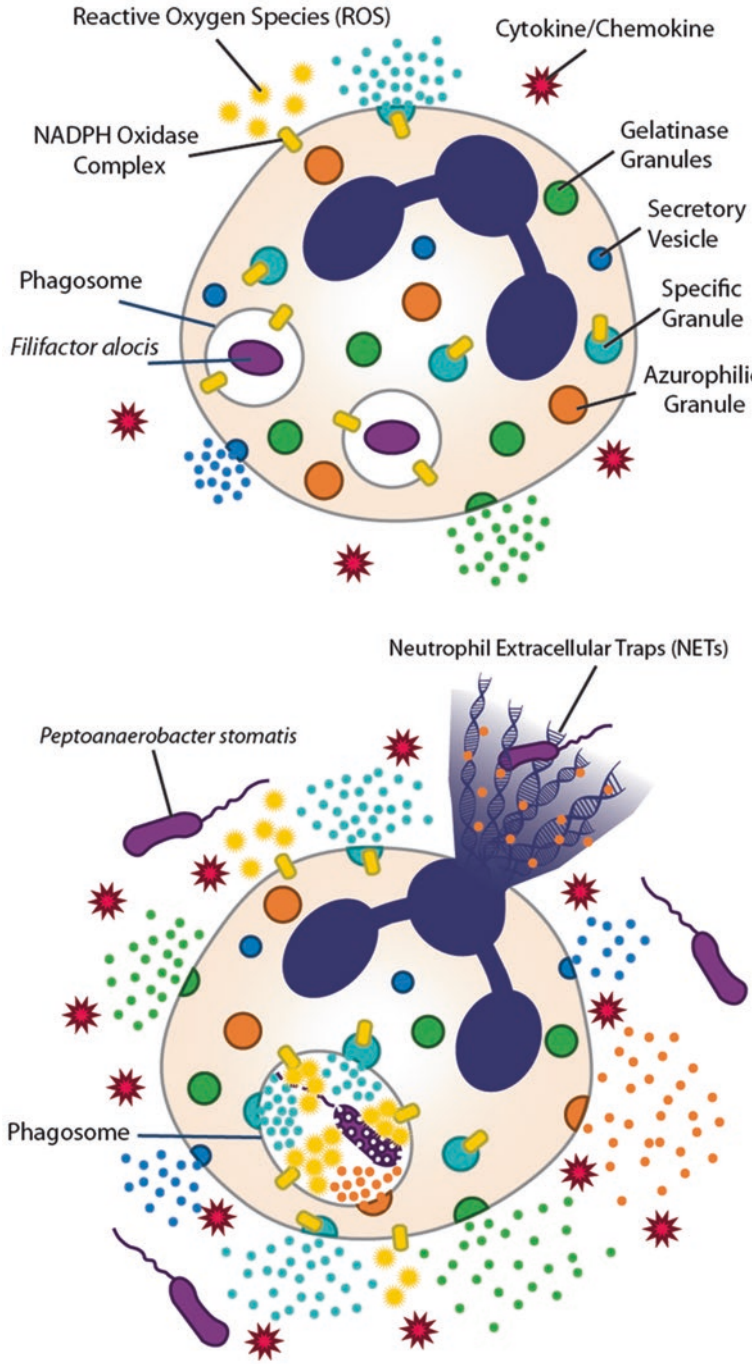

Fig. 1 Neutrophil interaction with *F. alocis* and *P. stomatis*. (**a**) Human neutrophils can efficiently phagocytize *F. alocis*. However, the oral pathogen is able to persist within the neutrophils by inducing minimal production of intracellular reactive oxygen species (ROS) and minimizing the fusion of antimicrobial granules with the bacteria-containing phagosome. Simultaneously, *F. alocis* stimulates a proinflammatory response from neutrophils by causing the moderate exocytosis of granules, release of proinflammatory cytokines, and small production of extracellular ROS. (**b**) Contrastingly, *P. stomatis* prevents phagocytosis by human neutrophils because once internalized is promptly eliminated. At the *P. stomatis* phagosome, there is robust generation of ROS and efficient fusion of the microbicidal specific and azurophilic granules to the phagosome. Despite its proficient killing, *P. stomatis* induces an extremely proinflammatory response form neutrophils. Upon *P. stomatis* challenge, neutrophils show an exaggerated exocytosis of all four granule subtypes, robust release of cytokines and chemokines, and induced the formation of neutrophil extracellular traps (NETs). Additionally, *P. stomatis* robustly primes neutrophils to produce an even greater extracellular ROS production in response to secondary stimulation

P. stomatis has the ability to promote NET formation, albeit to a lesser extent compared to *S. gordonii* (Armstrong et al. 2018). We recently reported that both *F. alocis* and *P. stomatis* trigger TLR2/6 signaling pathways, but the modulation of neutrophil-derived cytokines and chemokines by the two organisms is very different. Only *P. stomatis* challenge of human neutrophils, but not *F. alocis* or *P. gingivalis*, induced the release of active neutrophil-derived chemokines to promote both neutrophil and monocyte migration (Vashishta et al. 2019).

Based on the current knowledge about the interactions between neutrophils and the two emerging oral pathogens, *F. alocis* and *P. stomatis*, a model of the battlefield in the periodontal pocket is depicted in Fig. 2. Periodontal pathogens use different strategies to subvert and evade neutrophils killing while supporting inflammation. In the case of the emerging oral pathogens, *P. stomatis* and *F. alocis,* we see the story of the tortoise and the hare at play. When neutrophils encounter *P. stomatis*, they directly cause intense inflammation through the exocytosis of all four of their granules and production of ROS and NETs. *P. stomatis* also incites inflammation through the robust release of neutrophil-derived cytokines and chemokines. The chemokines will recruit more neutrophils and monocytes to the tissue, which can add to the inflammatory response. On the other hand, the cytokines and other inflammatory products like dead-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) will prime naïve neutrophils and heighten their response to any secondary stimulus, which will further magnify the inflammatory response. Ultimately, the hyperactive neutrophil response to the bacterial challenge results in the death of both *P. stomatis* and the neutrophil. Contrastingly, neutrophils' encounter with *F. alocis* is more moderate. Direct tissue damage still takes place through the *F. alocis*-induced exocytosis of three out of the 4 neutrophil granules with minimal ROS production, but since neutrophils are activated to a lesser degree, fewer cytokines and chemokines are released. In comparison to *P. stomatis, F. alocis* primes neutrophils to a lesser degree and does not recruit as many immune cells. The key to *F. alocis* pathogenesis is that it can remain viable within neutrophils and prolongs their lifespan (*I. Miralda, A. Vashishta, C. K. Klaes, R. J. Lamont, S. M. Uriarte, unpublished observations*). This outcome results in a delay in neutrophil clearance by macrophages, through efferocytosis, and inhibition of the resolution of inflammation.

Conclusion and Future Directions

High number of activated neutrophils are recruited to the gingival tissue but fail to clear the infection and instead contribute to sustain a dysregulated inflammation that provides a favorable environment for "inflammo-*philic*" pathogens to outgrow and promote immunopathology (Hajishengallis 2014). Significant advances have been made regarding the characterization of the complex microbial composition both in healthy as well as in disease sites. In the past decade, the metagenomics studies applied to subgingival plaque derived from periodontitis disease sites revealed the complex microbial composition. Furthermore, metatranscriptomic studies shed light into the complex functional interactions that occur within the periodontitis microenvironment. All these "omic" studies opened the door to a new field of the "yet-to-be cultivated" taxons, with high abundance in disease sites compared to healthy sites. The challenge now is to define what role these emerging oral pathogens play in disease progression and how they interact with the host immune response. What are the bacterial relationships between the established periodontal pathogens and the uncultivable or emerging oral bacteria? Do some of the emerging oral bacteria play a bystander role while others could be key players in promoting the shift in the bacterial composition? What are the potential virulence characteristics of these emerging oral organisms; have they evolved to evade or manipulate the host immune response? These are some of the open questions that will require investigation to advance our knowledge and understanding of this complex multifactorial chronic inflammatory disease.

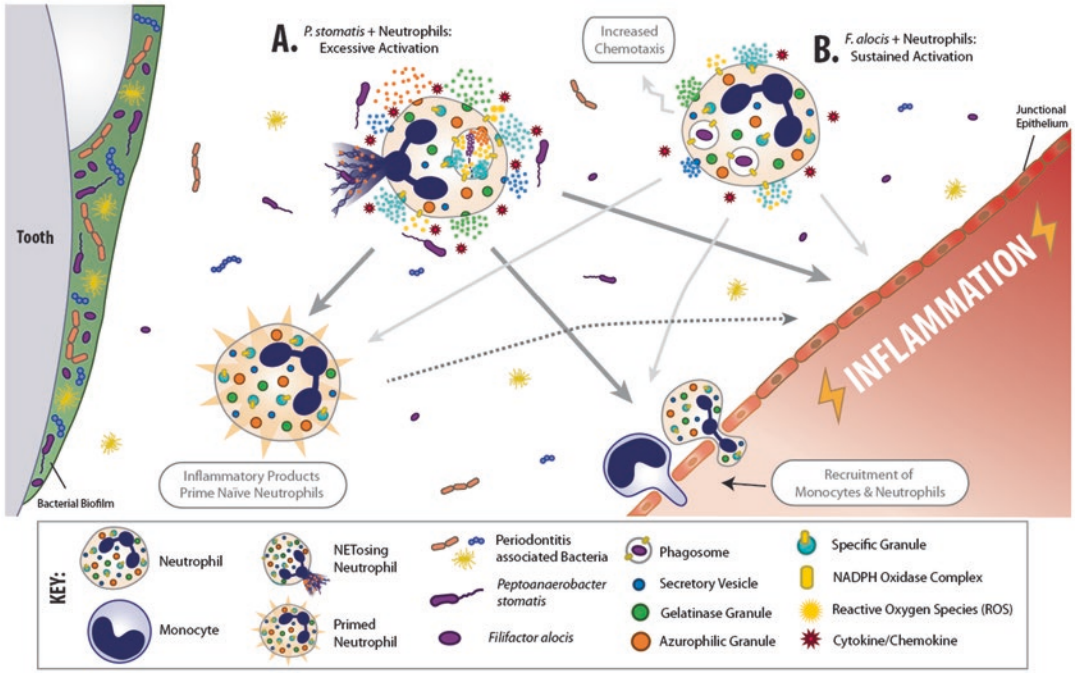

Fig. 2 The war zone of the gingival pocket: While neutrophils are essential to maintain homeostasis in the oral cavity, they can also drive inflammation and tissue destruction. Periodontal pathogens have different modus operandi against neutrophils to promote inflammation and contribute to the disease progression. (**a**) When neutrophils encounter *P. stomatis*, they directly cause intense inflammation through the exocytosis of all four of their granules and production of ROS and NETs. Furthermore, *P. stomatis* promotes robust release of neutrophil-derived cytokines and chemokines, which will recruit more neutrophils and monocytes to the tissue, inciting inflammation. Ultimately, the intense response provoked by the encounter results in the death of both *P. stomatis* and the neutrophil. (**b**) In stark contrast, neutrophils' encounter with *F. alocis* is more moderate. Direct tissue breakdown damage still takes place through the exocytosis of three out of the 4 neutrophil granule subtypes with minimal ROS production, but since neutrophils are activated to a lesser degree, fewer cytokines and chemokines are released. However, *F. alocis* increases the migration of neutrophils that have phagocytized the organism. The key to *F. alocis* pathogenesis is that the organism has developed strategies to subvert killing and remains viable within neutrophils. The effects of *P. stomatis*, *F. alocis*, and primed neutrophils are delineated with the bold dark gray arrows, the thin light gray arrows, and the dotted arrow, respectively

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